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ORIGINAL PAPER

Expression analysis of *Clavata1*-like and *Nodulin21*-like genes from *Pinus sylvestris* during ectomycorrhiza formation

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Abstract The ecology and physiology of ectomycorrhizal (EcM) symbiosis with conifer trees are well documented. In comparison, however, very little is known about the molecular regulation of these associations. In an earlier study, we identified three EcM-regulated *Pinus* expressed sequence tags (EST), two of which were identified as homologous to the *Medicago truncatula* nodulin *MtN21*. The third EST was a homologue to the receptor-like kinase *Clavata1*. We have characterized the expression patterns of these genes and of auxin- and mycorrhiza-regulated genes after induction with indole-3-butyric acid in *Pinus sylvestris* and in a time course experiment during ectomycorrhizal initiation with the co-inoculation of 2,3,5-triiodobenzoic acid, an auxin transport inhibitor. Our results suggest that different *P. sylvestris* nodulin homologues are associated with diverse processes in the root. The results also suggest a potential role of the *Clv1*-like gene in lateral root initiation by the ectomycorrhizal fungus.

Keywords Auxin · IBA · TIBA · *Pinus sylvestris* · *Laccaria bicolor* · Ectomycorrhiza · Symbiosis · *MtN21* · *Clavata1*-like

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Introduction

Symbiotic associations between tree roots and ectomycorrhizal fungi play important roles in boreal forests. Ectomycorrhizal fungi enhance the growth and fitness of trees by providing them with mineral and organic nutrients from the soil and by protecting the roots from pathogenic organisms in exchange for carbohydrates and organic compounds resulting from photosynthesis (Read 1991). In order to understand what characterizes the interaction between ectomycorrhizal (EcM) fungi and trees, a number of transcriptome studies has been carried out (Voiblet et al. 2001; Podila et al. 2002; Le Quere et al. 2005; Heller et al. 2008). Studies comparing the host responses to symbiotic, pathogenic, and saprotrophic interactions (Adomas et al. 2008) are also available. These studies have indicated that the number of mycorrhiza-specific genes in both partners may be relatively small. A characteristic of EcM interactions, which may explain these observations, is the morphogenetic differentiation in the plant root with the formation of a symbiotic organ in association with the fungal partner. As a result of colonization by EcM fungi, the tips of pine short roots may undergo dichotomous branching (Kaska et al. 1999). The molecular mechanisms regulating root development during interaction with the EcM fungus are still unclear (Felten et al. 2009, 2010). Phytohormones, especially auxins, play central role in EcM formation (Barker and Tagu 2000, and references therein; Jambois and Lapeyrie 2005; Felten et al. 2009). EcM fungi, e.g., *Laccaria bicolor* produce auxin (Ek et al. 1983) that can influence the root morphology and mycorrhization process (Gay et al. 1994). The interaction between auxin signaling and fungal volatile signals such as ethylene (Kaska et al. 1999; Splivallo et al. 2009; Felten et al. 2010) seems to be very important in this process.

In a previous study of the transcriptome in ectomycorrhizal development in *Pinus sylvestris* with *L. bicolor*, we have identified a set of expressed sequence tags (ESTs) representing Pine genes which were expressed at early stages in EcM formation (Heller et al. 2008). In the present study, we focused on three ESTs with a potential role in root morphogenesis. The first of these three ESTs showed similarity to *Clavata1* receptor-like kinase (RLK) genes of *Medicago truncatula* and *Arabidopsis* (*Clv1-like*). Several genes with homology to *Clavata1* RLK genes of *Arabidopsis* have been implicated in various types of organogenesis (Oka-Kira and Kawaguchi 2006; van Noorden et al. 2006). The remaining two are *MtN21-like-a* and *MtN21-like-b* clones showing similarity to an *MtN21* nodulin-like gene of *M. truncatula* and to *5NG4*, another member of the *Pinus* *MtN21* gene family (Busov et al. 2004). *5NG4* has been implicated in adventitious root formation (Busov et al. 2004), suggesting that especially the *MtN21-like-a* and *MtN21-like-b* ESTs could be suitable marker genes for the onset of lateral root formation in the *P. sylvestris*/*L. bicolor* symbiosis. In the present work, we studied the expression of these candidate genes during rhizogenesis and EcM development. We also compared the transcription of the genes under indole-3-butyric acid (IBA) and auxin transport inhibitor treatments to elucidate the role of auxin and auxin signaling in their regulation.

Materials and methods

Sequences and phylogenetic analysis

The conserved regions in the amino acid sequences of Pt *MtN21-like* (BG485740.1) and Pt *Clv1-like* (BF517399.1) and orthologous protein sequences (Shiu et al. 2004; Schnabel et al. 2005; Morillo and Tax 2006) were aligned using ClustalW. Thereafter, neighbor-joining trees were constructed. All the sequences used in this study can be found in Electronic supplementary material S1. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). Statistical support was estimated with a bootstrap analysis of 1,000 replicates.

Biological material

Four fungal isolates were used in the study: *L. bicolor* (Maire) Orton 238A (Finlay et al. 1992) and *Paxillus involutus* Maj (Gafur et al. 2004), which are capable of forming EcM *in vitro* with pine and birch, respectively, *Heterobasidion parviporum* FS6 (Karlsson et al. 2007), and *Phanerochete chrysosporium* RP78 (Stewart et al. 2000). *P. sylvestris* (Scots pine) seeds from Svenska

Skogsplantor (Saleby FP-45) were surface-sterilized with 33% hydrogen peroxide (H_2O_2) for 15 min, rinsed in several changes of distilled water, sown on 1% water agar Petri plates, and left to germinate under a photoperiod of 16 h light at a temperature of 18°C.

Preparation of the co-cultivation study

All fungi were cultivated on modified Melin-Norkrans (MMN) medium (Marx 1969) solidified with 1.5% agar. A 5-mm diameter agar plug was added to 15 mL of MMN medium in a 50-mL Falcon tube. After 14 days at 19°C, 14-day-old pine seedlings were transferred to tubes containing actively growing fungal colonies. Mock inoculations were prepared by transferring seedlings to 15 mL of MMN medium.

The co-cultivation experiment was incubated with a photoperiod of 16 h light at a temperature of 20°C. Three biological replicates were harvested at 0, 3, 6, 10, 24, 48, and 72 h post initiation of the experiment (hpi), flash-frozen in liquid nitrogen, and stored at −80°C until processed. All manipulations in the co-cultivation experiment were performed under axenic conditions.

EcM, auxin treatment, and spatial analysis of gene expression in *P. sylvestris*

Two-week-old *P. sylvestris* seedlings were transferred under axenic conditions to a hydroponic box system and were grown for 14 days in MMN medium devoid of glucose. Systems were kept at 21°C under a 16-h photoperiod.

To quantify the basal expression of the candidate genes, 1-month-old seedlings were harvested with RNAlater (Ambion). To facilitate the infiltration of RNAlater into the tissues, a light vacuum was applied for 2 min; thereafter, seedlings were dissected in RNAlater and the fractions of root primordia, root tips, hypocotyl, cotyledon, epicotyl, elongated root, intact shoot, and intact root were collected in two pooled representatives for each fraction.

For analysis of the effect of IBA on root morphogenesis and gene expression, *P. sylvestris* seedlings were treated with 100 μ M IBA (Saveen Werner). Three biological replicates, each of 100 seedlings, were harvested after 2, 8, 16, and 24 hpi.

In the EcM experiment, EcM fungal inoculum (*L. bicolor*) was grown for 21 days in liquid Hagem medium (Stenlid 1985), washed with sterile-distilled water, and homogenized for 60 s in a sterile blender. The homogenized mycelium was then added to the box systems. For treatments with *L. bicolor* and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA; Saveen Werner), 10 μ M of TIBA was added to the box system in combination with

L. bicolor. Control plants were grown with sterile MMN medium alone. Three biological replicates, each of 100 seedlings, were harvested for each treatment at 1, 5, 15, and 30 days post inoculation (dpi), flash-frozen in liquid nitrogen at harvest, and stored at -80°C until use.

In order to follow the physiological changes occurring after treatment with IBA, *L. bicolor* and the combination of *L. bicolor* and TIBA, the numbers of lateral roots, and the size of the main root were recorded for 100 seedlings per treatment over 30 days spanning the experiment and pictures were taken throughout the course of the experiment.

qRT-PCR analysis of gene transcription

Total RNA was extracted according to the methods of Chang et al. (1993) but samples less than 100 mg were homogenized directly in the extraction buffer. The final pellet was dissolved in RNase free water. One microgram of total RNA was digested with deoxyribonuclease I (Sigma-Aldrich). The RNA samples were reverse-transcribed with MMLV reverse transcriptase (Invitrogen) or the MMLV-derived enzyme iScript (Bio-Rad). All specific primer pairs were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Electronic supplementary material S2). The relative transcript abundance was detected using SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of template (concentration of template about 10 ng/ μL) and 0.5 or 0.75 μM of each primer depending on the optimization results. The program used was as follows: initial denaturation for 1 min at 95°C , followed by a cycle of 15 s at 95°C , 1 min at 60°C , repeated 40 times with a 5 min final extension at 60°C . After the completion of the protocol, samples were subjected to a melting curve analysis.

Relative expression levels were calculated using REST software (Pfaffl et al. 2002; Vandesompele et al. 2002) using two internal reference genes: *alpha-tubulin* (Karlsson et al. 2007) and *elongation factor 1a* (Electronic supplementary material S2). The amount of product generated by qRT-PCR from control roots at each time point was used as a reference sample. In the spatial distribution experiment, the intact root and shoot were used as well to establish the relative expression levels among the fractions in the shoot and the root. The results were expressed as the fold change of messenger RNA level over the reference. The effect of time points and treatments within gene studies were tested by one-way ANOVA, and the results were confirmed with non-parametrical Wilcoxon test. The differences between the time points for every studied gene were determined by Tukey–Kramer test. The confidence level of all tests was set to 0.05. Data analysis was performed with STATISTICA 8.0 (StatSoft Inc. USA).

Results

In *P. sylvestris*, lateral roots emerge about 5 to 6 weeks following seed germination without the addition of any exogenous factor. We observed that 4-week-old seedlings inoculated for 15 days with a homogenate of *L. bicolor* displayed lateral root formation.

At 30 dpi, the roots treated with *L. bicolor*, *L. bicolor* and TIBA, or IBA displayed distinctive features. Extension growth of the main root axis was reduced in the presence of the EcM fungus, and the number of lateral roots increased compared to the control (Supplementary Material S4). At 30 dpi, these lateral roots displayed the typical dichotomous branching of EcM pine roots and possessed a mantle of fungal mycelium (Electronic supplementary material S4). When *L. bicolor* was inoculated together with the addition of 10 μM of TIBA, an auxin transport inhibitor, roots were shortened but no lateral roots could be observed even though the fungus was growing near the roots (Electronic supplementary material S4). *P. sylvestris* roots treated with IBA had excessive numbers of lateral roots, which were much shorter in length compared to control roots (Electronic supplementary material S4).

Expression of the auxin-induced genes *GH3* and *iaa88*

The effect of IBA on the transcription levels of the genes coding for *P. sylvestris* *GH3* and *iaa88* genes led to 18- and 5-fold changes, respectively, when compared to the basal expression. Although the transcription levels were elevated throughout the experiment, they reached a significant peak at 8 h of treatment ($p>0.05$; Table 1). In the presence of the EcM fungus, the patterns observed were very different. The transcript levels for *GH3* were comparable to control at 5 dpi and decreased at 15 and 30 days ($p<0.05$). Transcript levels for *iaa88* decreased initially at day 1, and then gradually increased until day 15 (Fig. 2), after which they dropped to levels comparative to 1 dpi. Treatment with TIBA led to a transient increase of *GH3* at day 1 of the interaction with *L. bicolor*, but did not affect the *iaa88* transcription pattern ($p>0.05$; Fig. 2).

Expression of Nodulin-like genes (*MtN21-like-a/b* and *5NG4*)

The *Pinus taeda* *MtN21-like-a* and *MtN21-like-b* sequences showed a certain sequence overlap (seven amino acids, Supplementary Information S1 and S2) and they align within the same *P. taeda* EST contig (TC86303, DFCI Pinus Gene Indices; <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine>). This indicates that they originated from the same *P. taeda* gene and consequently the *PtMtN21-like* gene is referred to as *MtN21-like-a/b* unless

Table 1 Relative gene expression of *Pinus sylvestris* roots treated with indole-3-butyric acid (IBA)

Gene	2 h Fold change	8 h Fold change	16 h Fold change	24 h Fold change
<i>GH3</i>	4.2 (0.02)a	17.7 (0.06)b	13.3 (0.05)c	9.1 (0.19)c
<i>iaa88-like</i>	2.3 (0.04)a	5.0 (0.18)b	4.7 (0.11)bc	2.5 (0.14)ac
<i>MtN21-like-a</i>	1.7 (0.02)a	1.1 (0.01)a	1.6 (0.01)a	0.3 (0.01)b
<i>MtN21-like-b</i>	1.6 (0.02)a	0.6 (0.01)b	0.8 (0.03)b	0.2 (0.01)c
<i>5NG4</i>	1.0 (0.12)a	1.6 (0.23)b	2.5 (0.28)c	1.4 (0.17)b
<i>Clv1-like</i>	1.6 (0.02)a	2.2 (0.00)b	2.9 (0.07)b	1.8 (0.01)ab

Transcript levels were assessed by qPCR at 2, 8, 16, and 24 h of 100 μ M IBA treatment. The numbers represent the mean fold change of transcript levels compared to control roots. Values within brackets represent the standard deviation. Different letters indicate significant difference between the time points for each analyzed gene (Tukey–Kramer test, $p < 0.05$)

otherwise specified. The gene is a new member of the MtN21 gene family in *P. taeda*. Transcript levels of the *5NG4* and *MtN21-like-a/b* genes were higher in roots than the seedling as a whole in 1-month-old roots *P. sylvestris* seedlings (*MtN21-like-a/b* 6.6 times higher, $p = 0.05$; *5NG4* 3.2 times higher, $p = 0.066$). There is also a higher accumulation of *5NG4* and *MtN21-like-a/b* transcripts in regions with protruding root primordia (45- and 85-fold increase, respectively) compared to the intact plants. Co-cultivation with a 21-day-old *L. bicolor* colony led to a pronounced increase in *MtN21-like-a/b* transcript levels (about 28 times the control) after 24 h of contact with the colony ($p < 0.05$; Fig. 1). *P. involutus* induced a small increase in *MtN21-like-a/b* transcript levels at 12 and 24 hpi. In contrast, the orthologous gene with similarity to Pt5NG4, responded to all fungal isolates with elevated transcript levels at 12 hpi and throughout the experiment (Fig. 1).

In the presence of homogenized *L. bicolor*, the expression of the *MtN21-like-a/b* gene was repressed compared to mock-inoculated controls (Fig. 2). The addition of TIBA resulted in an induction of the *MtN21-like-a/b* gene at 5 and 30 dpi compared to the treatment with *L. bicolor* alone ($p < 0.05$). The patterns of gene expression observed for the *MtN21-like-a/b* and *5NG4* differed after treatment with the plant growth regulator IBA (Table 1). *MtN21-like-a/b* showed a small upregulation at 2 h, and at 24 h, a downregulation was seen while *5NG4* showed a 2.5 fold upregulation at 16 h ($p < 0.05$). Transcript levels of *5NG4* seem to increase after 5 days of co-cultivation with homogenized *L. bicolor* and decreased slightly at 15 and 30 dpi compared to the mock-inoculated control. Treatment with TIBA inhibits this regulation (Fig. 2).

Expression of the *Clavata-like* gene (*Clv1-like*)

The *Clv1-like* gene appears to be a member of the LRR-XI superfamily of RLKs in plants. However, the sequence was positioned basally with respect to angiosperm LRR-XI

(Electronic supplementary material S1). The gene was expressed at very low levels in *P. sylvestris*. In the experiment aiming to analyze the spatial distribution, the *Clv1-like* gene was close to the detection limit at about 10 copies at the start of the qPCR in all tissues except the root primordial, where 200–500 copies could be detected. The levels of *Clv1-like* gene expression remained under the

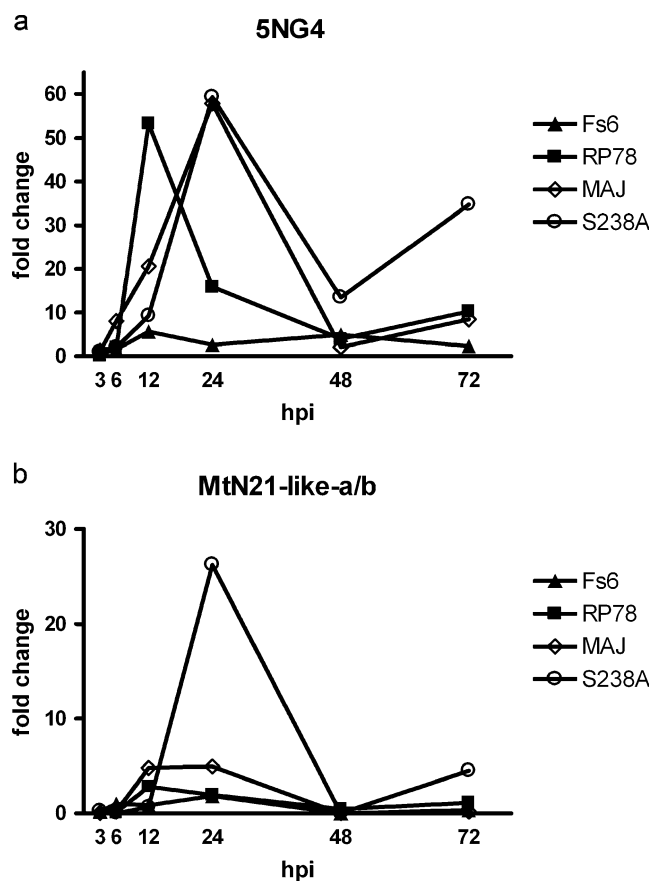


Fig. 1 Gene expression measured by qPCR of the **a** *5NG4* and **b** *MtN21-like-a/b* genes in *P. sylvestris* roots during the interaction with 21-day-old growing colonies of *H. annosum* (FS6, triangles), *P. chrysosporium* (RP78, squares), *P. involutus* (MAJ, diamonds), and with *L. bicolor* (S238A, circles)

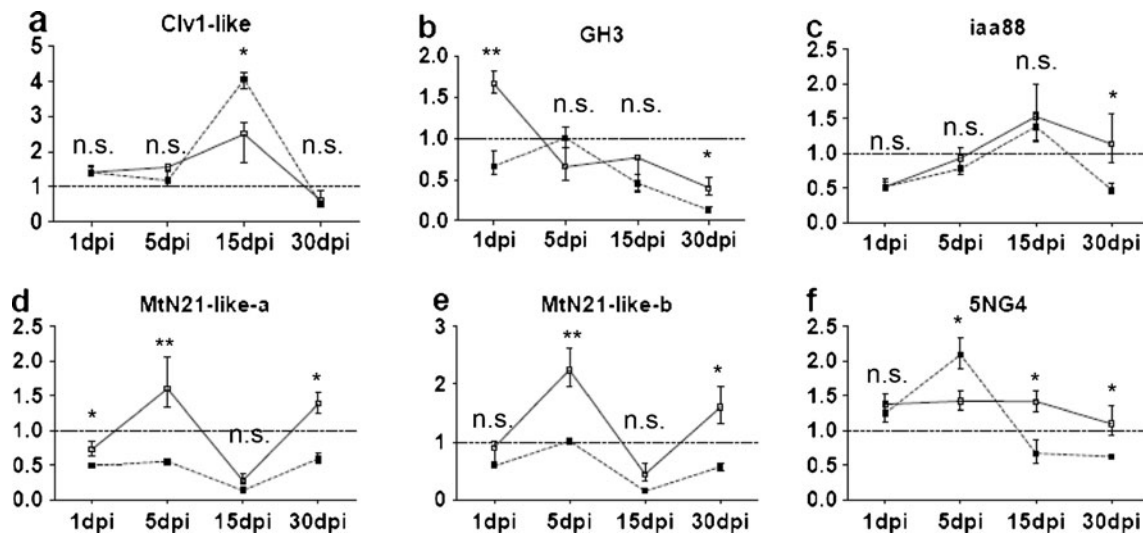


Fig. 2 Relative gene expression of **a** *Clv1-like*, **b** *GH3*, **c** *iaa88*, **d** *MtN21-like-a*, **e** *MtN21-like-b*, and **f** *5NG4* in *P. sylvestris* roots during interaction with the EcM fungus, *L. bicolor*. The relative fold change of the genes was assessed by qPCR at 1, 5, 15, and 30 days post inoculation in the presence (full connectors) or absence (dashed

connectors) of 2,3,5-triisobenzoic acid (TIBA). The average of fold changes of three biological replicates are presented for every time point, and error bars demonstrate standard errors. Statistical difference between the treatments within every time point is shown as follows: n.s. not significant, * $p < 0.05$, ** $p < 0.01$

detection limit in the co-cultivation experiment (data not shown). Treatment with IBA led to a relative increase in transcript level of the *Clv1-like* gene compared to the control at all time points during the 24-h monitoring period ($p < 0.05$; Table 1). In the presence of *L. bicolor*, the transcript levels of the *Clv1-like* gene rose to about 4-fold compared to the control from 5 to 15 dpi (Fig. 2). Addition of TIBA led to attenuated transcript accumulation levels at 15 dpi ($p < 0.05$; Fig. 2).

Discussion

We studied the expression of the candidate genes during rhizogenesis and EcM development under normal conditions as well as the influence of the auxin transport inhibitor TIBA. The application of auxin transport inhibitors has been reported to lead to local increase in auxin concentration in plant tissues through a blockage of the PIN1 cycling (Geldner et al. 2001). It has been shown that *L. bicolor* can induce lateral root formation in *Populus tremula* × *Populus tremuloides* and *Arabidopsis thaliana* even without physical contact. This induction can be completely inhibited by the auxin transport inhibitor 1-naphthylthalamic acid, showing that polar auxin transport is involved in lateral root formation in the early *L. bicolor*–plant interaction (Felten et al. 2009). We also observed an almost complete inhibition of *L. bicolor*-induced lateral root formation in our material after treatment with TIBA (Electronic supplementary material S4).

MtN21-like-a/b was identified through its upregulation during EcM formation (Heller et al. 2008), and just like the *P. taeda* gene *5NG4*, it shared homology with the *nodulin 21* gene from *M. truncatula* (Gamas et al. 1996). The two genes have a similar expression pattern in the untreated plant, with higher basal expression levels in roots than in shoots. However, in our system, the *P. sylvestris* homologues *5NG4* and *MtN21-like-a/b* did not respond similarly to fungal contact and colonization (Figs. 1 and 2). The *5NG4* gene is associated with adventitious root formation in *P. taeda* (Busov et al. 2004). Accordingly, the *P. sylvestris* *5NG4* transcript accumulated in root primordia. The *P. taeda* *5NG4* expression is induced by auxin in roots, stems, and hypocotyls (Busov et al. 2004) and the *P. sylvestris* homologue also responded to IBA treatment. Our results seem to emphasize the association of *5NG4* with local increases of auxin levels in developing tissues and a possible role in (adventitious) root formation as the gene responded to colonization by *L. bicolor* at 5 dpi, but application of TIBA inhibited the accumulation of *5NG4* transcripts at 5 dpi. The *MtN21-a* and *MtN21-b* clones correspond to a previously not studied member of the MtN21 family in *Pinus*. It has recently been shown that MtN21 homologues encode members of a transmembrane drug/metabolite exporter family and that the *Arabidopsis* mutant *Walls are thin1* encoding an MtN21 exhibit defect cell elongation (Ranocha et al. 2010). In *Pinus*, EcM colonization occurs primarily at short roots that have limited capacity to elongate, possibly because of peroxidase-mediated auxin degradation (Tarkka et al.

2001). We found that 24 h of IBA treatment and colonization with *L. bicolor* lead to lower steady-state levels of *MtN21-like-a/b* compared to untreated roots. Treatment with TIBA led to higher levels of *MtN21-like-a/b* expression compared to *L. bicolor* colonization alone. This indicates that inhibited auxin transport in roots may activate the expression of *MtN21-like-a/b* and that *5NG4* and *MtN21-like-a/b* are involved in different processes in the root, making *MtN21-like-a/b* and *5NG4* interesting candidates for more detailed functional and localization studies.

To validate the comparison of *5NG4* and *MtN21-like-a/b* expression patterns in response to *L. bicolor* and auxin treatment of *P. sylvestris* roots, the expression of two known auxin-regulated genes was also tested in our material. The upregulation of the auxin homeostasis gene *GH3* in *P. sylvestris* in the presence of IBA and the gradual down-regulation with *L. bicolor* colonization agree with the findings in *Pinus pinaster* where the transcript levels of *Pp-GH3.16* decreased gradually in the presence of the EcM fungus *Hebeloma cylindrosporum*, but increased in auxin treatments (Reddy et al. 2006). Our results confirm that the expression of plant genes associated with EcM formation is similar between closely related plant species. This is further underlined by the observation on the expression of *iaa88-like* genes in EcM colonization. We could detect expression of a *P. sylvestris iaa88-like* sequence with primers designed on the basis of two homologous genes, *Pp-iaa88* in *P. pinaster* and *PTIAA2* in *P. taeda* (Charvet-Candela et al. 2002; Goldfarb et al. 2003). The *Pp-iaa88* gene was reported to be highly expressed during fungal sheath formation (Charvet-Candela et al. 2002). Similarly, we found that the *P. sylvestris iaa88-like* sequence is upregulated at 15 dpi (Fig. 2), which also coincides with fungal sheath formation. TIBA application had no influence on the expression levels of *P. sylvestris iaa88-like* gene up to 15 dpi; however, it repressed the *P. sylvestris* gene significantly at 30 days, indicating that the induction of the *P. sylvestris iaa88-like* gene at 15 dpi is associated with the development of the symbiotic structures, supporting the concept of general EcM-associated transcription programs within the genus *Pinus*, possibly associated with organogenesis.

There are at least 38 ESTs with similarities to the LRR-XI superfamily of RLKs in the Pine EST databases. However, the expression pattern has been described for only one gene up to now (*PsRLK*, Avila et al. 2006). The LRR-XI RLK superfamily includes the well-known *Clavata1* gene of *Arabidopsis* (Shiu and Bleecker 2001) and the *M. truncatula* SUNN gene involved in autoregulation of nodulation (Oka-Kira and Kawaguchi 2006). The *Clv1-like* amino acid sequence clusters basally among the LRR-XI RLK superfamily (Electronic supplementary material Fig. 1). In

rhizobia symbiosis, SUNN controls the symbiosis, although it acts in the shoot (Oka-Kira and Kawaguchi 2006; van Noorden et al. 2006). On the basis of sequence similarity, it is tempting to suggest the possibility of an LRR-RLK-mediated autoregulatory mechanism in EcM formation as well, but the expression pattern of the *Clv1-like* gene suggests that the gene product is involved in other processes compared to other LRR-RLK members, which are active in aerial tissues. The *Clv1-like* gene showed higher expression levels in roots and root primordia compared to aerial tissues. The *Clv1-like* gene was induced by *L. bicolor* at 15 dpi in the EcM experiment, coinciding with lateral root and fungal sheath formation (Heller et al. 2008). The role of the *Clv1-like* gene in lateral root formation and fungal sheath formation deserves further exploration in future studies.

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